Hypercapnic ventilatory response is decreased in a mouse model of excessive erythrocytosis

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Abstract: The impact of cerebral Epo in the regulation of the hypercapnic ventilatory response (HcVR) is controversial. While we reported that cerebral Epo does not affect the central chemosensitivity in C57Bl6 mice receiving an intracisternal injection of sEpoR (the endogenous antagonist of Epo), a recent study in transgenic mice with constitutive high levels of human Epo in brain and circulation (Tg6) and in brain only (Tg21), showed that Epo blunts the HcVR, maybe by interacting with central and peripheral chemoreceptors. High Epo serum levels in Tg6 mice lead to excessive erythrocytosis (hematocrit about 80-90%), the main symptom of chronic mountain sickness (CMS). These latter results support the hypothesis that reduced central chemosensitivity accounts for the hypoventilation observed in CMS patients. To solve this intriguing divergence, we re-evaluate HcVR in Tg6 and Tg21 mouse lines, by assessing the metabolic rate (O2 consumption; and CO2 production), a key factor modulating ventilation, which effect was not considered in the previous study. Our results showed that the decreased HcVR observed in Tg6 mice (70% reduction; $p<0.01$) was due to a significant decrease in the metabolism (40%; $p<0.0001$) rather than Epo's effect on CO2 chemosensitivity. Additional analysis in Tg21 mice did not revealed differences of HcVR or metabolism. We concluded that cerebral Epo does not modulate the central chemosensitivity system, and that a metabolic effect upon CO2 inhalation is responsible for decreased HcVR observed in Tg6 animals. As CMS patients also show decreased HcVR, our findings might help to better understand respiratory disorders at high altitude.

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Hypercapnic ventilatory response is decreased in a mouse model of excessive erythrocytosis

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Running title: response to hypercapnia matters in chronic mountain sickness

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ABSTRACT

The impact of cerebral Epo in the regulation of the hypercapnic ventilatory response (HcVR) is controversial. While we reported that cerebral Epo does not affect the central chemosensitivity in C57Bl6 mice receiving an intracisternal injection of sEpoR (the endogenous antagonist of Epo), a recent study in transgenic mice with constitutive high levels of human Epo in brain and circulation (Tg6) and in brain only (Tg21), showed that Epo blunts the HcVR, maybe by interacting with central and peripheral chemoreceptors. High Epo serum levels in Tg6 mice lead to excessive erythrocytosis (hematocrit about 80-90%), the main symptom of chronic mountain sickness (CMS). These latter results support the hypothesis that reduced central chemosensitivity accounts for the hypoventilation observed in CMS patients. To solve this intriguing divergence, we re-evaluate HcVR in Tg6 and Tg21 mouse lines, by assessing the metabolic rate ($\dot{V}_{O_2}$ = O_2 consumption; and $\dot{V}_{CO_2}$ = CO_2 production), a key factor modulating ventilation, which effect was not considered in the previous study. Our results showed that the decreased HcVR observed in Tg6 mice (~70% reduction; $p<0.01$) was due to a significant decrease in the metabolism (~40%; $p<0.0001$) rather than Epo's effect on CO_2 chemosensitivity. Additional analysis in Tg21 mice did not revealed differences of HcVR or metabolism. We concluded that cerebral Epo does not modulate the central chemosensitivity system, and that a metabolic effect upon CO_2 inhalation is responsible for decreased HcVR observed in Tg6 animals. As CMS patients also show decreased HcVR, our findings might help to better understand respiratory disorders at high altitude.

(252 words)

INTRODUCTION

Excessive erythrocytosis is a main symptom of chronic mountain sickness (CMS, or Monge´s disease), a clinical syndrome that affects people who reside at altitudes higher than 2,500 m above sea level. It is estimated that about 5–10% of the world's population living at high-altitude may develop this illness (27, 43). In the Andes of Bolivia and Peru (> 4,000m), CMS affects 15-20% of the adult male population, and its prevalence increases with age, rising up to 30% by the age of 50 (25, 31). Chronic hypoxemia and neurological symptoms (such as headache, fatigue, somnolence, and alterations of sleep and memory) (28, 36) are also associated to CMS. Moreover, excessive erythrocytosis leads to life-threatening cyanosis, hyperemia, increased blood viscosity, thrombosis, and pulmonary hypertension (45). Remarkably, despite hypoxemia, it has been suggested that the basal ventilation and the hypoxic ventilatory response of CMS patients compared to healthy dwellers at same altitudes are reduced (28, 36). This observation led to the hypothesis that the ability of carotid bodies to sense the blood's PaO_2 in CMS patients is blunted (28, 36). Moreover, there are reports suggesting that the CO_2 chemosensitivity in CMS patients is also decreased, as they show a blunted hypercapnic ventilatory response (HcVR) compared to non-affected highlanders (40). In line, as the work of Leon-Velarde et al, showed no differences in the hypoxic ventilatory response between healthy highlanders and CMS patients, it was suggested that chronic hypoxemia, excessive erythrocytosis and CMS should be associated to a diminished CO_2 chemosensitivity, rather than a reduced hypoxic ventilatory response (26). It was also observed that in the course of the disease CMS patients develop a time-dependent increase of hemoglobin, accompanied by elevated $PaCO_2$ and reduced of PaO2 (32). Moreover, hypoxia caused a greater increase in total CO_2 sensitivity in sea level than in the high altitude and CMS subjects combined (11). Similar results were also reported previously by Fatemian et al., showing that total (but nor peripheral) chemosensitivity to CO_2 in hypoxia was higher in subjects at sea level compared to high altitude CMS patients (12). Indeed, sensing $PaCO_2$ provides a strong excitatory input to the respiratory rhythm generator through activation of chemoreceptors in the brainstem (33) and in the carotid bodies (13). Even a small increase in arterial PCO_2 evokes a robust increase in the respiratory activity: in humans a 1mmHg increase in $PaCO_2$ may lead to a 2L/min increase in lung ventilation (42). Consequently, it was also postulated that hypoventilation observed in CMS patients might be secondary to reduced sensitivity to CO_2, and as such CO_2 sensitivity as an important factor in the etiology of this disease.

We previously reported that - apart from its role in erythropoiesis - Epo is a potent stimulator of the hypoxic ventilatory response by interacting with respiratory centers in the brainstem (1, 41). On the other hand, by blocking the Epo's activity in the brainstem of adult C57Bl6 mice by intracisternal injections of the soluble Epo receptor (sEpoR), we reported that Epo reduced the basal minute ventilation by ~25%, but did not affect the central chemosensitivity (2). In contrast, a recent study using transgenic mice (Tg6: Epo overexpression in brain and circulation; Tg21: Epo overexpression in brain), suggested that Epo, presumably by interacting with central and peripheral respiratory chemocenters, blunts the HcVR (29). As such, the relevant question whether Epo decreases (or not) the CO_2 chemosensitivity is debated.

Moreover, as Tg6 mice show excessive erythrocytosis (hematocrit about 80-90%), the Tg6 strain is an interesting animal model to investigate if Epo and/or excessive erythrocytosis leads to hypoventilation in CMS patients due to reduced sensitivity to CO_2. Thus, in this work we re-evaluated the hypercapnic ventilation of Tg6 and Tg21 mice, and additionally assessed the metabolic rate ($\dot{V}_{O_2}$ = O_2 consumption and $\dot{V}_{CO_2}$ = CO_2 production), a key factor modulating ventilation, which effect was neglected in the previous study performed with these transgenic mice. Our working hypothesis was that when animals are exposed to hypercapnic conditions the decreased HcVR reported in Tg6 is due to changes in the metabolism, rather than due to Epo's modulation of the neural chemosensitivity. Indeed, the literature reports that hypercapnia alters $\dot{V}_{O_2}$, and proposes that numerous factors may contribute to such metabolic alteration (23). In line with these observations our results suggest that cerebral Epo does not alter the central CO_2 chemosensitivity and that the increased amount of circulating Epo, and the resulting excessive erythrocytosis may be crucial factors mediating a decreased metabolic response under CO_2 stimulation, which in turn will blunt the HcVR.

MATERIAL & METHODS

Animals

Detailed description of Tg6 and Tg21 strains were reported previously (37, 47). In brief, both transgenic strains are bread in a C57BL/6 back ground. The Tg6 mouse line showed increased Epo levels in lungs (12-folds / WT) and brain (26-folds / WT), accompanied by a doubled hematocrit value (Fig 1) (19). Half of the offspring were hemizygous for the transgene, while the other half were WT and thus were used as control animals (WT-Tg6) (44). Similarly, Tg21 heterozygotes mice, overexpressing Epo in the brain (4-folds/WT; Fig 1), were backcrossed with C57Bl/6 mice for more than six generations to obtain the corresponding control mice (WT-Tg21) used in this study. Here we used adult (2 - 3 months age) male Tg6 (hemizygous), Tg21 (homozygous) and corresponding controls. Animal experiments were approved by the Laval University Animal Ethics Committee (protocol #12-119-1) and carried out in accordance with the standards and guidance of the Canadian Council on Animal Care.

Respiratory recordings using whole-body plethysmography

Set-up details.

We used whole body plethysmography to record respiratory frequency (f_R), tidal volume (V_T), and minute ventilation ($\dot{V}_E = f_R \times V_T$). Minute ventilation is defined as the volume of inhaled or exhaled gas per minute. Basal minute ventilation is the minute ventilation under normocapnic/normoxic conditions, while ventilatory response to hypercapnia is the difference between the basal minute ventilation and the minute ventilation recorded under hypercapnic stimulation. Animals were place into a 600 ml plethysmograph chamber (Emka, Technologies, Paris, France) continuously supplied with fresh air (around 180 $ml.min^{-1}$) at room temperature. A differential pressure transducer (Emka technologies) was connected between the recording chamber and the built-in reference chamber. A single injection of 500 µl of air inside the chamber was used for calibration of the flow trace. A subsampling pump was used to draw (~50-75 ml/min) a sample of outflowing air for analysis of respiratory gases. In the outflowing line water pressure and CO_2 levels were measured with dedicated gas analysers (respectively RH 300 - Sable Systems International, Las Vegas, NV, USA - and CD-3A - AEI technologies, Pittsburgh, PA, USA). Oxygen levels in the inflowing and outflowing gas lines were measured with a dual channel O_2 analyzer (S3-A/II - AEI Technologies). All signals were acquired and recorded on a computer using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK), and used offline to calculate respiratory frequency (f_R), tidal volume (V_T), minute ventilation ($\dot{V}_E = f_R \times V_T$), CO_2 production ($\dot{V}_{CO_2}$), and O_2 consumption rate ($\dot{V}_{O_2}$). Body weight was measured routinely after every experiment to express the tidal volume in ml/100g. Body temperature was measured at the beginning and at the end of the experiment with a rectal thermocouple probe (Physitemp).

Recordings and signal analysis

After the animal familiarized within the plethysmography chamber (about 2 hours), basal ventilation was recorded at normocapnia (air) for 10 to 20 minutes. Hypercapnia was achieved by using a gas mixture containing 21% O_2 , 5% CO_2, in N_2, and the recording lasted 10 min.

During baseline recordings, the subsampling pump was connected to the inflowing gas line for 2 to 3 minutes. This procedure was used to verify that the two channels of the O_2 analyzer read similar values in the inflowing and outflowing gas lines, and to record the values of inflowing CO_2 and H_2O required for the calculation of metabolic rate (see below).

Respiratory frequency and inspiratory volume were calculated breath-by-breath using a custom script in Spike 2, then values of baseline ventilation were determined on the portions of the recording during which the breathing pattern was stable and regular, with the lowest respiratory frequency. During hypercapnia we selected fragments of stable and regular breathing patterns. For metabolic rate, we used the portions of the recordings where CO_2 in the outflowing gas line displayed the lowest value.

The section of the calibrated flow trace corresponding to inspiration was integrated by the software, and the corresponding volume was corrected by using the standard equation described for whole body plethysmography (4). Tidal volume (VT), respiratory frequency (fR), minute ventilation ($\dot{V}_E$), oxygen consumption ($\dot{V}_{O_2}$) and CO_2 production ($\dot{V}_{CO_2}$) were calculated as previously described (1) by using standard equations.

Quantification of hematocrit and Epo

At the end of experimentation mice were anaesthetized by an intra-peritoneal injection of a mixture of ketamine (80 mg kg^{-1}) and xylazine (10 mg kg^{-1}). Blood samples were drawn by cardiac puncture into heparinized Ependorff tubes (for plasma collection after samples centrifugation at 18 000 **g**, 15 min**;** Heraeus Instruments Biofuge 15, USA) and from Ependorff tubes into capillary tubes, to assess the hematocrit percentage (after samples centrifugation at 20 000 RCF, 10 min; American scientific products, Haemofuge 1216, USA). In following, brain samples were obtained after transcardial perfusion of mice with phosphate buffer (0.1 **m**, pH7.4) and immediately frozen in liquid nitrogen. Total brain and plasma Epo concentration was determined using ^{125}I-Epo-based radioimmunoassay (RIA-DiaSorin, Stillwater, MN, USA), accordingly to previous published protocols (Wenger et al., 1998; Soliz, et al., 2005). The lower detection limit for RIA was 4 U/l, the intra-assay/interasay variances was < 2% and <6%, respectively. All Epo determinations were performed in duplicate.

Statistical analysis

The differences between groups were evaluated with a two-way ANOVA for repeated measurement using groups as the independent variable and hypercapnia as the repeated variable. If the ANOVA gave

significant effects for groups, hypercapnia, or significant groups by hypercapnia interaction, we used a Fisher's LSD post-hoc analysis to test the effects of groups at each level of hypercapnia, or the effects of hypercapnia within each group. All analysis and graphs were done with the GraphPad prism 6.0 software (La Jolla, CA, USA). The reported values are means ± SEM. Differences were considered significant at two-tailed: *p <0.05 and ***p<0.0001.

RESULTS

Tg6 mice show a marked reduction of the hypercapnic ventilatory response (HCVR)

As expected, compared to WT-Tg6 control animals, Tg6 mice showed increased levels of hematocrit and Epo in plasma and brain (Fig 1 and table 1). Ventilatory ($\dot{V}_E$, fR and VT) and metabolic ($\dot{V}_{O_2}$, $\dot{V}_{CO_2}$) parameters were evaluated in transgenic Tg6 and WT-Tg6 control mice under basal normocapnic conditions. Neither ventilatory ($\dot{V}_E$, fR and VT) nor metabolic ($\dot{V}_{O_2}$, $\dot{V}_{CO_2}$) parameters were found to be different between strains under a normocapnic environment (Table 1 and Fig 2A-E). However, once exposed to hypercapnia, Tg6 showed a 70% reduction in ventilatory response (Fig 2A) as compared to control mice. This decreased ventilatory response was due to a significant reduction of the VT (Fig 2C) and no changes in fR (Fig 2B). Moreover, the evaluation of metabolism under hypercapnia showed a significant decreased $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ in transgenic mice in comparison to control WT-Tg6 (Fig 2D,E). As result, the respiratory equivalent ratio for O_2 ($\dot{V}_E/\dot{V}_{O_2}$) or CO_2 ($\dot{V}_E/\dot{V}_{CO_2}$) was similar in control and Tg6 mice (Table 1). These data demonstrate that the blunted ventilation observed in Tg6 mice under hypercapnia, is due to a significant reduction of the metabolism rather than the impact of Epo in the neural chemoregulation to CO_2 (Fig 2 F-G).

Tg21 mice, overexpressing Epo in brain only, do not show altered hypercapnic ventilatory response

As our previous experiments do not allow differentiating between the impact of Epo in central or peripheral centers controlling ventilation, in a next step we used our phethysmography system to evaluated the hypercapnic ventilatory response in Tg21 transgenic mice (and corresponding control mice) overexpressing Epo only in the brain only (Fig 1C). Our results showed that under normocapnic and hypercapnic conditions both the ventilatory ($\dot{V}_E$, VT, fR), and metabolic ($\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$) parameters were similar between transgenic and control animals (Table 2 and Fig 3A-E). Accordingly, $\dot{V}_E/\dot{V}_{O_2}$ and $\dot{V}_E/\dot{V}_{CO_2}$ are similar between strains (Fig 3F,G). These results are in line with our previous report in which the intracisternal injections of the endogenous Epo antagonist (the soluble Epo receptor, sEpoR), despite decreasing basal ventilation, did not affected the central chemosensitivity (2). All together, our present and

previous results strongly suggest that cerebral Epo does not affect the central chemosensitivity.

DISCUSSION

In the present study we investigated the HcVR of a transgenic mouse line (Tg6) that shows high levels of Epo in brain and plasma, and an excessive erythrocytosis similar to the observed in altitude dwellers suffering from chronic mountain sickness (CMS). Interestingly, as occurring in CMS patients, Tg6 mice significantly decrease their HcVR. Additional measurements of HcVR performed in a second line of transgenic mice overexpressing Epo in the brain only (Tg21), confirmed that cerebral Epo does not account for the neural modulation of central CO_2 chemosensitivity. On the contrary, our results showed that a reduced metabolism (O_2 consumption and CO_2 production) is responsible of the decreased hypercapnic ventilation obtained in Tg6 mice. We concluded that circulating Epo overexpression and excessive erythrocytosis might be crucial factors enhancing the magnitude of the metabolic depression induced by CO_2 inhalation.

Previous studies performed on mice in our laboratory have shown that Epo and its receptor are widely distributed in the brainstem respiratory groups (41). Moreover we reported that cerebral Epo activates the hypoxic ventilatory response by interacting with the respiratory centers in the brainstem. In vitro experiments performed in brainstem-spinal cord preparations (en bloc technique) supported these results by showing that Epo is indeed a key regulator of oxygen homeostasis of the central respiratory network during both normoxia and hypoxia (1, 8-10, 24). On the other hand, as the brainstem is the main structure containing the CO_2 chemosensors (17, 18, 34), it was also hypothesized that cerebral Epo might in addition modulate the ventilatory response to hypercapnia. However, so far, the results obtained in our and other laboratories have shown contradictory results. By performing an intracisternal injection (cisterna Magna) of the endogenous antagonist of Epo (the sEpoR), we showed that cerebral Epo does not affect the central CO_2 chemosensitivity of C57BL6 mice (2). However, a recent study in which Tg6 and Tg21 mice were used, suggests that Epo decreases the HcVR, maybe by interacting with the central and peripheral chemoreceptors (29). To solve this divergence, here we re-evaluate the HcVR in Tg6 and Tg21 animal strains by additionally measuring the metabolism ($\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$). Indeed, the literature reveals contradictory effects of hypercapnia on $\dot{V}_{O_2}$. Increase $\dot{V}_{O_2}$ and ventilation was observed in ponies, the former being attributed to the energetic cost of hyperventilation (23), no effect on $\dot{V}_{O_2}$ was reported in rats exposed to 2 and 5% CO_2 (3, 39), and decreased $\dot{V}_{CO_2}$ was described in cats exposed to 4% CO_2 (38). Finally, although the mechanisms involved are not well understood, it is know that hypercapnia elicits hypothermia in a number of vertebrates (3). As such, although this response seems to be widespread among mammals, still little is known about the impact of metabolic regulation on the hypercapnic stimulation of ventilation (7). We reasoned that, showing considerable physiological changes associated with the regular oxygen usage and energy production (such as doubled hematocrit, doubled hemoglobin concentration, high blood viscosity, tripled amount of systemic NO, massive increase of plasma and

cerebral Epo), Tg6 mice appear to adjust their metabolism to cope with extreme respiratory challenges such hypercapnia. Accordingly to this hypothesis our results showed that Tg6 mice elicited a 70% reduction of the HcVR, in association with a severe reduction of the metabolism ($\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$). As the ventilatory differences between transgenic Tg6 and control animals disappeared when the ventilatory equivalent ratio for O_2 ($\dot{V}_E/\dot{V}_{O_2}$) and CO_2 ($\dot{V}_E/\dot{V}_{CO_2}$) were obtained, these results clearly suggest that the drastic HcVR depression of Tg6 mice was mediated by decreased metabolism. Furthermore, as these results are not conclusive in regard of the impact of central (and/or peripheral) Epo in the response to hypercapnia, we used a second transgenic mouse line, overexpressing Epo in the brain only (Tg21). Tg21 mice compared to corresponding control animals showed similar HcVR, metabolism, and ventilatory equivalent ratio to O_2 and CO_2 ($\dot{V}_E/\dot{V}_{O_2}$ and $\dot{V}_E/\dot{V}_{CO_2}$). Contrary to these results, the previous study in which HcVR was evaluated in transgenic mice, reported a decreased HcVR in Tg21 mice (29). However the published data show large variability and a p-value in the limit to allow the data to be considered as significantly different (p=0.043). On the contrary, our data shows small variability and a p-value far to the limit of significance (p=0.4754). In addition, more compelling is the fact that our current results are in line with our previous reported data in which central chemosensitivity was unaffected in C57Bl6 mice intra-cisternaly treated with sEpoR (2). Of note, in current and previous (2) study animals were of 2-3 months of age, which certainly accounts for reducing the data variability. All together, our results support the notion that cerebral Epo is not implicated in the modulation of the central CO_2 chemosensitivity, and that the decreased HcVR of Tg6 mice is mediated by a reduction in the metabolic drive, as can clearly be observed in the figure 1F&G.

Regarding mechanisms, it seems important to recall that Tg6 animals show an increased constitutive expression of eNOS associated with a three-fold increased level of nitric oxide (NO) (37). Such NO augmentation prevents Tg6 mice from developing hypertension, stroke, myocardial infarction, or thrombolembolism. Moreover, when L-NAME (a inhibitor of NOS) was administrated by the drinking water, all treated Tg6 mice died after 52h (37). Apart however from its effects on vascular relaxation and antithrombotic functions (14, 20, 35), NO is an inhibitory messenger in the carotid body activity (46). Moreover, there are several pieces of evidence showing that NO is implicated in the regulation of the body temperature (3, 21). It was observed that the inhibition of NO in horses reduced by ~70% their sweat during exercise (22, 30), and NO is involved in the central mechanism of thermoregulation during fever in rabbits (16). Interestingly, the level of circulating NO is also increased (by 25-50%) at high altitude in Tibetans and Aymaras (from Bolivia) compared to the low altitude reference (5). As such, the augmentation of NO seems to be dependent on the need of vascular relaxation (as occurring in the erythrocythosis-mediated increase of blood density), and on the level of circulating Epo, as it has been reported that Epo induces NO production in microvascular endothelial cells of lungs (6, 15). Taken all this

evidences together, it is appealing to postulate that excessive erythrocytosis and overexpression of circulating Epo leads to NO overproduction reported in Tg6 mice. In turn, NO by modulating the carotid body activity, and by reducing the metabolism might contribute to the decreased ventilatory response of Tg6 mice exposed to CO_2.

Perspectives and significance

Due to excessive erythrocytosis, Tg6 animals mimic some conditions of altitude dwellers suffering from CMS. However, as Tg6 mice also overexpress Epo in the brain, and neural Epo is a powerful ventilatory stimulant under normoxic and hypoxic condition (2, 41), these animals do not show baseline hypoventilation. Moreover, our previous work showed that the hypoxic ventilatory response of Tg6 animals was similar to control mice, and that the ventilatory response to hypoxic in transgenic mice was due to a remarkable increase of the respiratory frequency (most probably mediated by the overexpression of neural Epo), compensated by a significant decrease of VT. However, as our results suggest that neural Epo does not impact the central chemosensitivity, the Tg6 strain is a good model to study the HcVR of CMS patients. As such, it is tempting to speculate that the increased level of Epo availability (due to the reduced amount of plasmatic sEpoR (43)), and the excessive erythrocytosis occurring in CMS patients, may lead to an exaggerated production of circulating NO. Such NO imbalance, apart from inducing a vascular relaxation, may also contribute to hypoventilation and reduced ventilatory response to hypoxia and hypercapnia, by its action on the metabolism and in the inhibition of the carotid body activity. The relevance of our results is that - for the first time – convincing evidence is provided that decreased metabolism is responsible for the reduced ventilatory response to increased PaCO2 of CMS subjects. Further investigation in humans subjects are required to test this hypothesis, and also to determine if NO is the associated mechanism implicated.

In summary our results show that the decreased HcVR of Tg6 mice is mediated by a significant reduction of the hypercapnic metabolism. Moreover, our data are in line with our previous report showing that neural Epo does not impact the central chemosensitivity. Finally, our results suggest that plasma Epo and excessive erythrocytosis could be major players in the observed reduction of the hypercapnic metabolism, possibly by producing an exaggerated production of circulating NO.

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Figure legends

Table 1. Comparison of ventilatory and metabolic parameters between control and Tg6 mice under baseline normocapnic and hypercapnic conditions. Animals per group: Tg6 = 9; Ctrl = 7.

Table 2. Comparison of ventilatory and metabolic parameters between control and Tg21 mice under baseline normocapnic and hypercapnic conditions. Animals per group: Tg6 = 7; Ctrl = 6.

Figure 1. Basal parameters in transgenic and contraol mice. Hematocrit (A) and Epo concentrations in plasma (pg/ml) (B) and brain (mU Epo/mg protein) (C) of transgenic (Tg6 and Tg21) mice and corresponding control animals. *p<0.01, Tg21 vs. Ctrl; ***p<0.0001 Tg6 vs. Ctrl. Animals per group: Tg6 = 10; WT-Tg6 = 7 & Tg21 = 11; WT-Tg21 = 8.

Fig 2. Hypercapnic ventilatory response in Tg6 mice is drastically reduced. A) Minute-ventilation ($\dot{V}_E$), B) respiratory frequency (fR), C) tidal volume (VT), D) O_2 consumption ($\dot{V}_{O_2}$), E) CO_2 production ($\dot{V}_{CO_2}$), F) minute ventilation in response to O_2 metabolism ($\dot{V}_E$ vs $\dot{V}_{O_2}$), and G) the respiratory equivalent ratio for O_2 ($\dot{V}_E/\dot{V}_{O_2}$) or CO_2 ($\dot{V}_E/\dot{V}_{CO_2}$) were recorded in male Tg6 and control (WT-Tg6) mice under normocapnic basal conditions and hypercapnia of 5%CO_2. Dashed lines represents the values of $V_E/\dot{V}_{O_2}$ (panel F) or $V_E/\dot{V}_{CO_2}$ (panel G) reported for mice each normocapnic and hypercapnic levels respectively from bottom to top. All values are mean ± sem; ***p<0.0001, Tg6 vs. Ctrl at hypercapnia; animals per group = 9 – 7.

Fig 3. Hypercapnic ventilatory response is not affected in Tg21 mice. A) Minute-ventilation ($\dot{V}_E$), B) respiratory frequency (fR), C) tidal volume (VT), D) O_2 consumption ($\dot{V}_{O_2}$), E) CO_2 production ($\dot{V}_{CO_2}$), F) minute ventilation in response to O_2 metabolism ($\dot{V}_E$ vs $\dot{V}_{O_2}$), and G) the respiratory equivalent ratio for O_2 ($\dot{V}_E/\dot{V}_{O_2}$) or CO_2 ($\dot{V}_E/\dot{V}_{CO_2}$) was similar in control and Tg6 mice were recorded in male Tg21 and control (WT-Tg21) mice under normocapnic basal conditions and hypercapnia of 5%CO_2. Dashed lines

represents the values of $V_E/\dot{V}_{O_2}$ (panel F) or $V_E/\dot{V}_{CO_2}$ (panel G) reported for mice each normocapnic and hypercapnic levels respectively from bottom to top. All values are mean ± sem; animals per group = 7 – 6.

References

1. **Ballot O, Joseph V, and Soliz J**. Endogenous brain erythropoietin is a potent sex-specific respiratory stimulant in adult and newborn mice. *J Appl Physiol (1985)* jap 00143 02015, 2015.
2. **Ballot O, Laouafa S, Elliot-Portal E, Tam R, Voituron N, Joseph V, and Soliz J**. The central chemosensitivity is not altered by cerebral erythropoietin. *Neurosci Lett* 609: 63-68, 2015.
3. **Barros RC, Oliveira ES, Rocha PL, and Branco LG**. Respiratory and metabolic responses of the spiny rats Proechimys yonenagae and P. iheringi to CO2. *Respir Physiol* 111: 223-231, 1998.
4. **Bartlett D, Jr., and Tenney SM**. Control of breathing in experimental anemia. *Respir Physiol* 10: 384-395, 1970.
5. **Beall CM, Laskowski D, Strohl KP, Soria R, Villena M, Vargas E, Alarcon AM, Gonzales C, and Erzurum SC**. Pulmonary nitric oxide in mountain dwellers. *Nature* 414: 411-412, 2001.
6. **Beleslin-Cokic BB, Cokic VP, Wang L, Piknova B, Teng R, Schechter AN, and Noguchi CT**. Erythropoietin and hypoxia increase erythropoietin receptor and nitric oxide levels in lung microvascular endothelial cells. *Cytokine* 54: 129-135, 2011.
7. **Branco LG, and Wood SC**. Role of central chemoreceptors in behavioral thermoregulation of the toad, Bufo marinus. *Am J Physiol* 266: R1483-1487, 1994.
8. **Caravagna C, Kinkead R, and Soliz J**. Post-natal hypoxic activity of the central respiratory command is improved in transgenic mice overexpressing Epo in the brain. *Respir Physiol Neurobiol* 200: 64-71, 2014.
9. **Caravagna C, Schneider-Gasser EM, Ballot O, Joseph V, and Soliz J**. Chronic overexpression of cerebral Epo improves the ventilatory response to acute hypoxia during the postnatal development. *Int J Dev Neurosci* 2015.
10. **Caravagna C, and Soliz J**. PI3K and MEK1/2 molecular pathways are involved in the erythropoietin-mediated regulation of the central respiratory command. *Respir Physiol Neurobiol* 206: 36-40, 2015.
11. **Fatemian M, Gamboa A, Leon-Velarde F, Rivera-Ch M, Palacios JA, and Robbins PA**. Selected contribution: Ventilatory response to CO2 in high-altitude natives and patients with chronic mountain sickness. *J Appl Physiol (1985)* 94: 1279-1287; discussion 1253-1274, 2003.
12. **Fatemian M, Nieuwenhuijs DJ, Teppema LJ, Meinesz S, van der Mey AG, Dahan A, and Robbins PA**. The respiratory response to carbon dioxide in humans with unilateral and bilateral resections of the carotid bodies. *J Physiol* 549: 965-973, 2003.

13. **Forster HV, Martino P, Hodges M, Krause K, Bonis J, Davis S, and Pan L**. The carotid chemoreceptors are a major determinant of ventilatory CO2 sensitivity and of PaCO2 during eupneic breathing. *Adv Exp Med Biol* 605: 322-326, 2008.

14. **Furchgott RF, and Zawadzki JV**. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288: 373-376, 1980.

15. **Genc S, Kuralay F, Genc K, Akhisaroglu M, Fadiloglu S, Yorukoglu K, Fadiloglu M, and Gure A**. Erythropoietin exerts neuroprotection in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated C57/BL mice via increasing nitric oxide production. *Neurosci Lett* 298: 139-141, 2001.

16. **Gourine AV**. Pharmacological evidence that nitric oxide can act as an endogenous antipyretic factor in endotoxin-induced fever in rabbits. *Gen Pharmacol* 26: 835-841, 1995.

17. **Guyenet PG, Stornetta RL, Abbott SB, Depuy SD, Fortuna MG, and Kanbar R**. Central CO2 chemoreception and integrated neural mechanisms of cardiovascular and respiratory control. *J Appl Physiol* 108: 995-1002, 2010.

18. **Guyenet PG, Stornetta RL, and Bayliss DA**. Retrotrapezoid nucleus and central chemoreception. *J Physiol* 586: 2043-2048, 2008.

19. **Heinicke K, Baum O, Ogunshola OO, Vogel J, Stallmach T, Wolfer DP, Keller S, Weber K, Wagner PD, Gassmann M, and Djonov V**. Excessive erythrocytosis in adult mice overexpressing erythropoietin leads to hepatic, renal, neuronal, and muscular degeneration. *Am J Physiol Regul Integr Comp Physiol* 291: R947-956, 2006.

20. **Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, and Fishman MC**. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 377: 239-242, 1995.

21. **Jackson DC, Palmer SE, and Meadow WL**. The effects of temperature and carbon dioxide breathing on ventilation and acid-base status of turtles. *Respir Physiol* 20: 131-146, 1974.

22. **Joyner MJ**. Invited editorial on "Nitric oxide and thermoregulation during exercise in the horse". *J Appl Physiol (1985)* 82: 1033-1034, 1997.

23. **Kaminski RP, Forster HV, Bisgard GE, Pan LG, Dorsey SM, and Barber BJ**. Effects of altered ambient temperature on metabolic rate during CO2 inhalation. *J Appl Physiol (1985)* 58: 1592-1596, 1985.

24. **Khemiri H, Seaborn T, Gestreau C, and Soliz J**. Erythropoietin and soluble erythropoietin receptor regulate the neural control of hypoxic respiration in newborn mice. *Respir Physio Neurobiol* 183: 151-121, 2011.

25. **Leon-Velarde F, and Arregui A**. Desadaptación a la vida en las grandes alturas. *Instituto Francés de Estudios Andinos (IFEA)* 1994.

26. **Leon-Velarde F, Gamboa A, Rivera-Ch M, Palacios JA, and Robbins PA**. Selected contribution: Peripheral chemoreflex function in high-altitude natives and patients with chronic mountain sickness. *J Appl Physiol (1985)* 94: 1269-1278; discussion 1253-1264, 2003.

27. **Leon-Velarde F, Maggiorini M, Reeves JT, Aldashev A, Asmus I, Bernardi L, Ge RL, Hackett P, Kobayashi T, Moore LG, Penaloza D, Richalet JP, Roach R, Wu T, Vargas E, Zubieta-Castillo G, and Zubieta-Calleja G**. Consensus statement on chronic and subacute high altitude diseases. *High Alt Med Biol* 6: 147-157, 2005.

28. **Leon-Velarde F, and Richalet JP**. Respiratory control in residents at high altitude: physiology and pathophysiology. *High Alt Med Biol* 7: 125-137, 2006.

29. **Menuet C, Khemiri H, de la Poeze d'Harambure T, and Gestreau C**. Polycythemia and high levels of erythropoietin in blood and brain blunt the hypercapnic ventilatory response in adult mice. *Am J Physiol Regul Integr Comp Physiol* 310: R979-991, 2016.

30. **Mills PC, Marlin DJ, Scott CM, and Smith NC**. Nitric oxide and thermoregulation during exercise in the horse. *J Appl Physiol (1985)* 82: 1035-1039, 1997.

31. **Monge CC, Arregui A, and Leon-Velarde F**. Pathophysiology and epidemiology of chronic mountain sickness. *Int J Sports Med* 13 Suppl 1: S79-81, 1992.

32. **Moore LG, Niermeyer S, and Vargas E**. Does chronic mountain sickness (CMS) have perinatal origins? *Respir Physiol Neurobiol* 158: 180-189, 2007.

33. **Nattie E**. CO2, brainstem chemoreceptors and breathing. *Prog Neurobiol* 59: 299-331, 1999.

34. **Nattie E, and Li A**. Central chemoreception is a complex system function that involves multiple brain stem sites. *J Appl Physiol* 106: 1464-1466, 2009.

35. **Panza JA, Quyyumi AA, Brush JE, Jr., and Epstein SE**. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med* 323: 22-27, 1990.

36. **Reeves JT, and Leon-Velarde F**. Chronic mountain sickness: recent studies of the relationship between hemoglobin concentration and oxygen transport. *High Alt Med Biol* 5: 147-155, 2004.

37. **Ruschitzka FT, Wenger RH, Stallmach T, Quaschning T, de Wit C, Wagner K, Labugger R, Kelm M, Noll G, Rulicke T, Shaw S, Lindberg RL, Rodenwaldt B, Lutz H, Bauer C, Luscher TF, and Gassmann M**. Nitric oxide prevents cardiovascular disease and determines survival in

447 polyglobulic mice overexpressing erythropoietin. *Proc Natl Acad Sci U S A* 97: 11609-11613,
448 2000.
449 38. **Sachdeva U, and Jennings DB**. Effects of hypercapnia on metabolism, temperature, and
450 ventilation during heat and fever. *J Appl Physiol (1985)* 76: 1285-1292, 1994.
451 39. **Saiki C, and Mortola JP**. Effect of CO2 on the metabolic and ventilatory responses to
452 ambient temperature in conscious adult and newborn rats. *J Physiol* 491 (Pt 1): 261-269, 1996.
453 40. **Severinghaus JW, Bainton CR, and Carcelen A**. Respiratory insensitivity to hypoxia in
454 chronically hypoxic man. *Respir Physiol* 1: 308-334, 1966.
455 41. **Soliz J, Joseph V, Soulage C, Becskei C, Vogel J, Pequignot JM, Ogunshola O, and**
456 **Gassmann M**. Erythropoietin regulates hypoxic ventilation in mice by interacting with brainstem
457 and carotid bodies. *J Physiol* 568: 559-571, 2005.
458 42. **Spyer KM, and Gourine AV**. Chemosensory pathways in the brainstem controlling
459 cardiorespiratory activity. *Philos Trans R Soc Lond B Biol Sci* 364: 2603-2610, 2009.
460 43. **Villafuerte FC, Macarlupu JL, Anza-Ramirez C, Corrales-Melgar D, Vizcardo-Galindo G,**
461 **Corante N, and Leon-Velarde F**. Decreased plasma soluble erythropoietin receptor in high-
462 altitude excessive erythrocytosis and Chronic Mountain Sickness. *J Appl Physiol (1985)* 117:
463 1356-1362, 2014.
464 44. **Vogel J, and Gassmann M**. Adaptive mechanisms in mice constitutively overexpressing
465 erythropoietin. *International Congress Series* 1275: 63-70, 2004.
466 45. **Vogel J, Kiessling I, Heinicke K, Stallmach T, Ossent P, Vogel O, Aulmann M, Frietsch T,**
467 **Schmid-Schonbein H, Kuschinsky W, and Gassmann M**. Transgenic mice overexpressing
468 erythropoietin adapt to excessive erythrocytosis by regulating blood viscosity. *Blood* 102: 2278-
469 2284, 2003.
470 46. **Wang ZZ, Stensaas LJ, Dinger BG, and Fidone SJ**. Nitric oxide mediates chemoreceptor
471 inhibition in the cat carotid body. *Neuroscience* 65: 217-229, 1995.
472 47. **Wiessner C, Allegrini PR, Ekatodramis D, Jewell UR, Stallmach T, and Gassmann M**.
473 Increased cerebral infarct volumes in polyglobulic mice overexpressing erythropoietin. *J Cereb*
474 *Blood Flow Metab* 21: 857-864, 2001.

Table 1

Tg6 mice

Parameter	Group	Condition		Effect		
		normocapnia mean ± SEM	hypercapnia mean ± SEM	Group	Condition	Interaction
Minute ventilation	WT-Tg6	148.0±21.4	434.8±58.8	F=7.8 p=0.011	F=39.6 p=0.0001	F=7.8 p=0.01
	Tg6	164.0±42.4	254.5±28.5			
Respiratory frequency	WT-Tg6	226.2±32.6	321.6±21.5	F=3.07 p=0.092	F=19.1 p=0.0002	F=1.002 p=0.3
	Tg6	218.2±29.5	263.0±14.5			
Tidal volume	WT-Tg6	0.7±0.1	1.3±0.1	F=7.7 p=0.01	F=46.3 p=0.0001	F=8.9 p=0.006
	Tg6	0.7±0.1	0.9±1.0			
O_2 consumption	WT-Tg6	4.2±0.14	3.8±0.2	F=18.9 p=0.0009	F=39.1 p=0.0001	F=13.8 p=0.003
	Tg6	4.1±0.1	2.7±0.1			
CO_2 production	WT-Tg6	2.5±0.1	2.5±0.1	F=24.5 p=0.0003	F=48.6 p=0.0001	F=59.2 p=0.0001
	Tg6	2.7±0.1	1.4±0.1			
$\dot{V}E/\dot{V}O_2$	WT-Tg6	34.9±4.3	104.2±15.8	F=0. p=0.4	F=47.6 p=0.0001	F=2.0 p=0.2
	Tg6	39.2±9.2	97.5±15.3			
$\dot{V}E/\dot{V}CO_2$	WT-Tg6	0.6±0.01	155.6±20.2	F=0.5 p=0.5	F=34.6 p=0.0001	F=0.5 p=0.5
	Tg6	0.7±0.01	183.2±27.8			
hematocrit	WT-Tg6	44.9±1.0		P=0.8		
	Tg6	78.4±1.4				
plasma Epo	WT-Tg6	33.7±4.9		p=0.4		
	Tg6	415.4±9.3				
brain Epo	WT-Tg6	11.6±2.1		p=0.1		
	Tg6	182.6±8.4				

Table 2

Tg21 mice

Parameter	Group	Condition		Effect		
		normocapnia mean ± SEM	hypercapnia mean ± SEM	Group	Condition	Interaction
Minute ventilation	WT-Tg21	149.6±18.8	376.8±30.1	F=0.9 p=0.3	F=53.8 p=0.0001	F=0.002 p=1.0
	Tg21	174.8±18.6	349.2±35.7			
Respiratory frequency	WT-Tg21	227.2±25.8	318.8±12.1	F=3.0 p=0.1	F=27.1 p=0.0001	F=0.4 p=0.6
	Tg21	208.8±7.9	282.0±13.6			
Tidal volume	WT-Tg21	0.8±0.03	1.2±0.1	F=0.0006 p=1.0	F=30.0 p=0.0001	F=0.3 p=0.6
	Tg21	0.7±0.1	1.2±0.08			
O_2 consumption	WT-Tg21	4.4±0.1	3.7±0.01	F=0.6 p=0.4	F=9.9 p=0.005	F=0.03 p=0.9
	Tg21	4.2±0.3	3.6±0.2			
CO_2 production	WT-Tg21	2.8±0.1	2.4±0.07	F=2.1 p=0.2	F=7.7 p=0.01	F=0.2 p=0.6
	Tg21	2.6±0.2	2.3±0.07			
$\dot{V}E/\dot{V}O_2$	WT-Tg21	64.0±5.6	108.5±7.1	F=2.1 p=0.2	F=16.9 p=0.0008	F=0.7 p=0.4
	Tg21	58.7±9.3	87.9±10.3			
$\dot{V}E/\dot{V}CO_2$	WT-Tg21	64.0±5.6	168.9±11.4	F=2.1 p=0.2	F=52.1 p=0.0001	F=1.0 p=0.3
	Tg21	58.7±9.3	137.5±16.3			
hematocrit	WT-Tg21	46.5±1.1		p=0.7		
	Tg21	45.6.4±1.7				
plasma Epo	WT-Tg21	33.7±4.9		p=0.001		
	Tg21	415.4±9.3				
brain Epo	WT-Tg21	11.6±2.1		p=0.001		
	Tg21	182.6±8.4				

Figure1

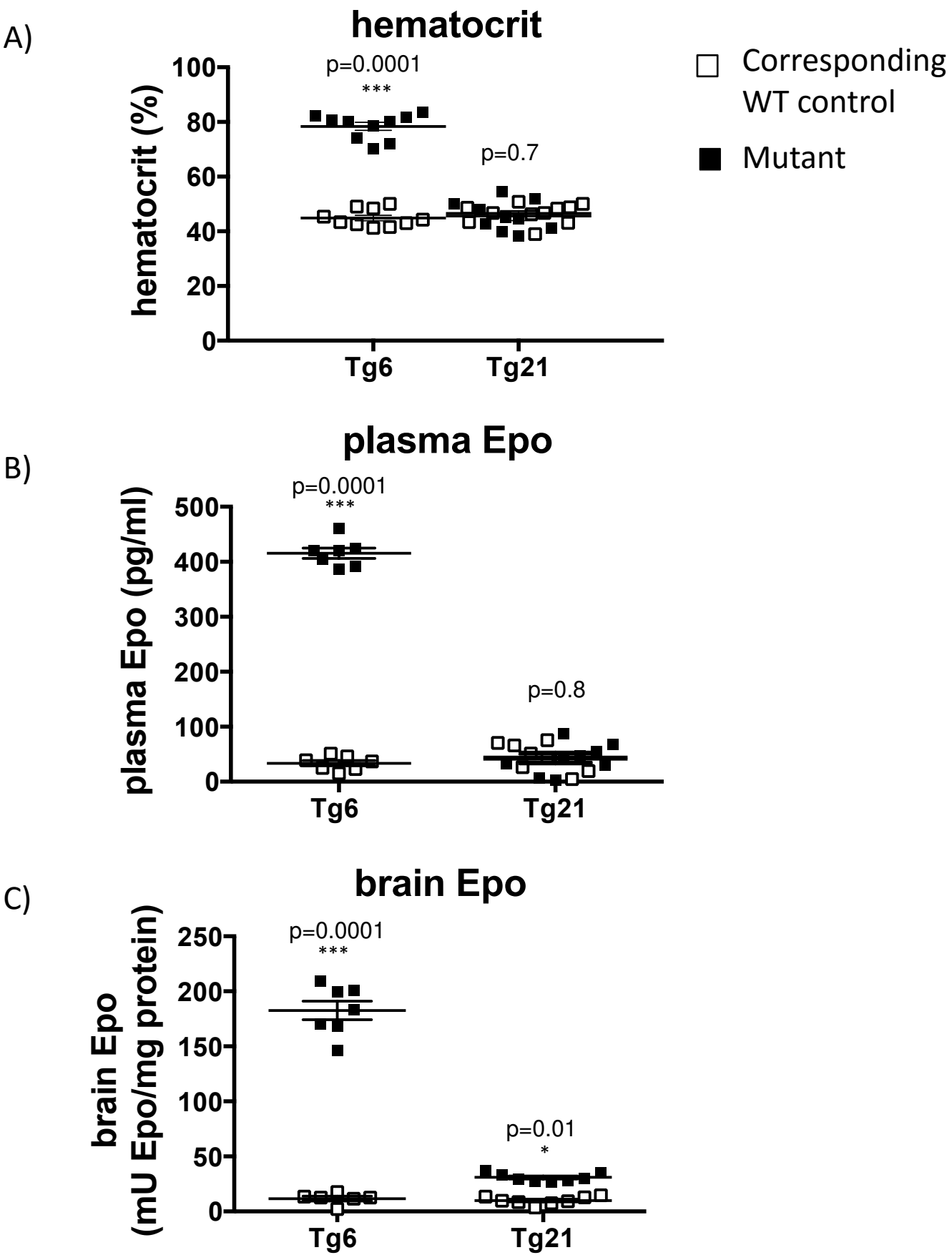

A)
hematocrit
p=0.0001
***
p=0.7
hematocrit (%)
100
80
60
40
20
0
Tg6
Tg21
Corresponding WT control
Mutant
B)
plasma Epo
p=0.0001
***
p=0.8
plasma Epo (pg/ml)
500
400
300
200
100
0
Tg6
Tg21
C)
brain Epo
p=0.0001
***
p=0.01
*
brain Epo (mU Epo/mg protein)
250
200
150
100
50
0
Tg6
Tg21

Figure 2

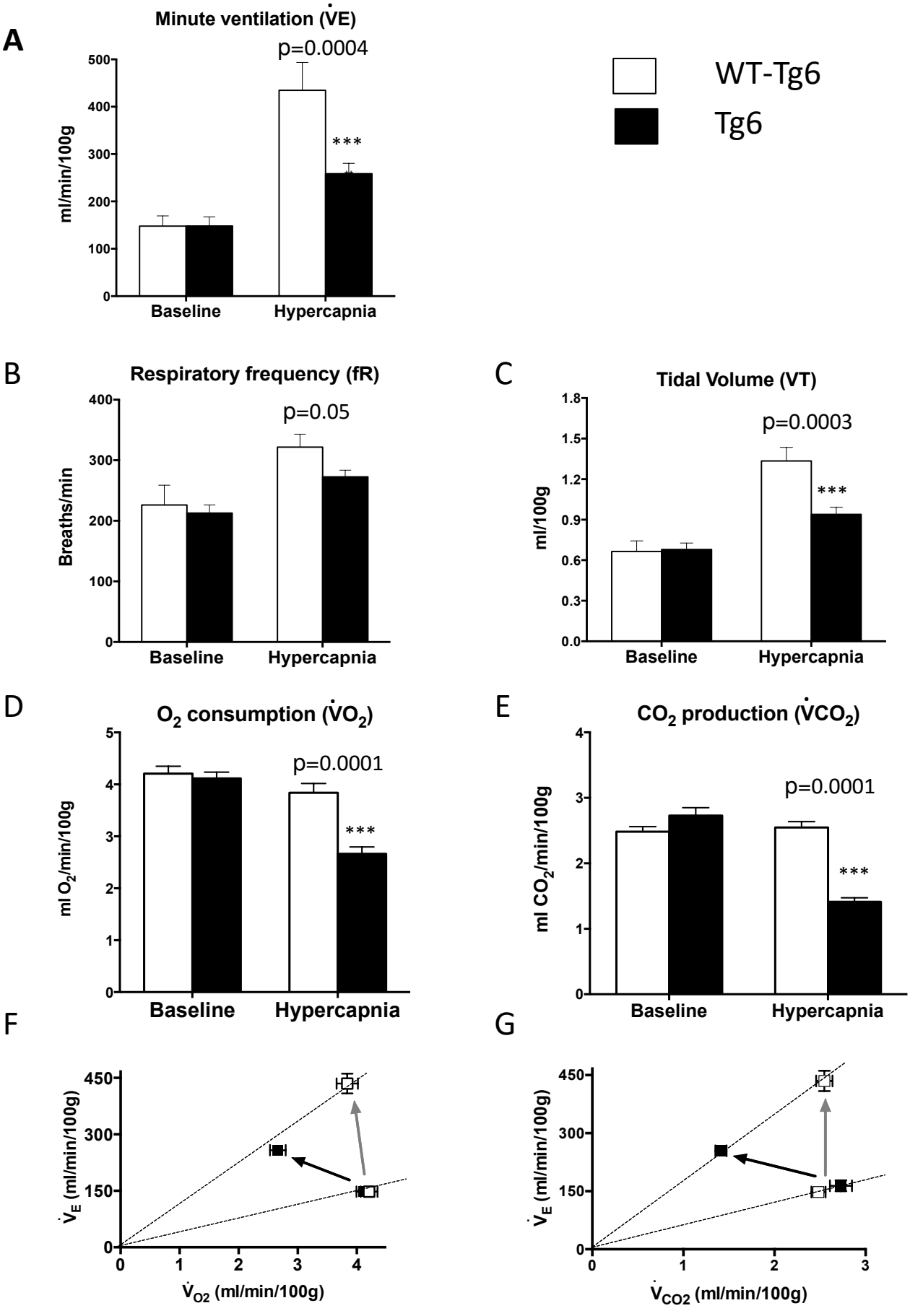
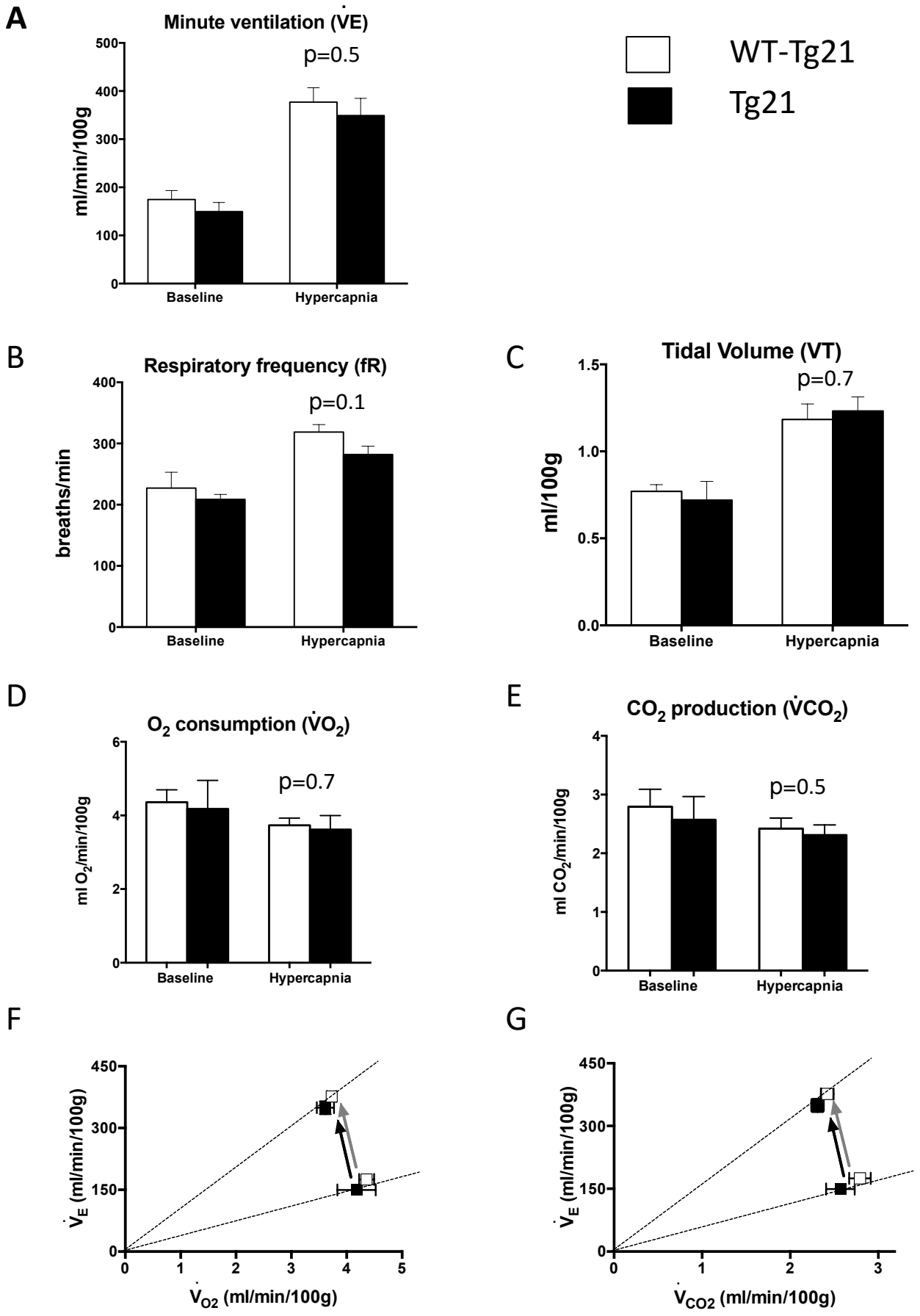

A
Minute ventilation (V̇E)
p=0.0004
***
ml/min/100g
500
400
300
200
100
0
Baseline
Hypercapnia
WT-Tg6
Tg6
B
Respiratory frequency (fR)
p=0.05
Breaths/min
400
300
200
100
0
Baseline
Hypercapnia
C
Tidal Volume (VT)
p=0.0003
***
ml/100g
1.8
1.5
1.2
0.9
0.6
0.3
0.0
Baseline
Hypercapnia
D
O2 consumption (V̇O2)
p=0.0001
***
ml O2/min/100g
5
4
3
2
1
0
Baseline
Hypercapnia
E
CO2 production (V̇CO2)
p=0.0001
***
ml CO2/min/100g
4
3
2
1
0
Baseline
Hypercapnia
F
V̇E (ml/min/100g)
450
300
150
0
0
1
2
3
4
V̇O2 (ml/min/100g)
G
V̇E (ml/min/100g)
450
300
150
0
0
1
2
3
V̇CO2 (ml/min/100g)

Figure 3


A
Minute ventilation (V̇E)
p=0.5
ml/min/100g
500
400
300
200
100
0
Baseline
Hypercapnia
WT-Tg21
Tg21
B
Respiratory frequency (fR)
p=0.1
breaths/min
400
300
200
100
0
Baseline
Hypercapnia
C
Tidal Volume (VT)
p=0.7
ml/100g
1.5
1.0
0.5
0.0
Baseline
Hypercapnia
D
O2 consumption (V̇O2)
p=0.7
ml O2/min/100g
6
4
2
0
Baseline
Hypercapnia
E
CO2 production (V̇CO2)
p=0.5
ml CO2/min/100g
4
3
2
1
0
Baseline
Hypercapnia
F
V̇E (ml/min/100g)
450
300
150
0
0
1
2
3
4
5
V̇O2 (ml/min/100g)
G
V̇E (ml/min/100g)
450
300
150
0
0
1
2
3
V̇CO2 (ml/min/100g)